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Neuropathic pain from an experimental neuritis of the rat sciatic nerve

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Abstract

Painful peripheral neuropathies involve both axonal damage and an inflammation of the nerve. The role of the latter by itself was investigated by producing an experimental neuritis in the rat. The sciatic nerves were exposed at mid-thigh level and wrapped loosely in hemostatic oxidized cellulose (Oxycel™) that on one side was saturated with an inflammatory stimulus, carrageenan (CARRA) or complete Freund's adjuvant (CFA), and on the other side saturated with saline. In other rats, a myositis was created by implanting Oxycel saturated with CFA into a pocket made in the biceps femoris at a position adjacent to where the nerve was treated. Pain-evoked responses from the plantar hind paws were tested before treatment and daily thereafter. Statistically significant heat- and mechano-hyperalgesia, and mechano- and cold-allodynia were present on the side of the inflamed nerve (CARRA or CFA) for 1–5 days after which responses returned to normal. There were no abnormal pain responses on the side of the saline-treated nerve, and none in the rats with the experimental myositis. The abnormal pain responses were inhibited by *N*-methyl-D-aspartate receptor blockade with MK-801, but were relatively resistant to the dose of morphine tested (10 mg/kg). Light microscopic examination of CARRA-treated nerves, harvested at the time of peak symptom severity, revealed that the treated region was mildly edematous and that there was an obvious endoneurial infiltration of immune cells (granulocytes and lymphocytes). There was either a complete absence of degeneration, or the degeneration of no more than a few tens of axons. Immunocytochemical staining for CD4 and CD8 T-lymphocyte markers revealed that both cell types were present in the epineurial and endoneurial compartments. The endoneurial T-cells appeared to derive from the endoneurial vasculature, rather than from migration across the nerve sheath.

We conclude that a focal inflammation of the sciatic nerve produces neuropathic pain sensations in a distant region (the ipsilateral hind paw) and that this is not due to axonal damage. The neuropathic pain is specific to inflammation of the nerve because it was absent in animals with the experimental myositis and in those receiving sham-treatment. These results suggest that an acute episode of neuritis-evoked neuropathic pain may contribute to the genesis of chronically painful peripheral neuropathies, and that a chronic (or chronically recurrent) focal neuritis might produce neuropathic pain in the absence of significant (or clinically detectable) structural damage to the nerve. The model that we describe is likely to be useful in the study of the neuroimmune factors that contribute to painful peripheral neuropathies.

Keywords: Carrageenan; Complete Freund's adjuvant; Neuropathic pain; Sciatic nerve

1. Introduction

The peripheral neuropathies that are accompanied by neuropathic pain sensations are usually thought to include cases of trauma (e.g. causalgia and phantom pain), postherpetic neuralgia and painful diabetic neuropathy. The occurrence of abnormal pain sensations in conjunction with many other peripheral neuropathies is considered less frequently. Nevertheless, neuropathic pain sensations are known to occur in a very wide variety of conditions (Scadding, 1994). For example, neuropathic pain is reported in multiple sclerosis, Guillain–Barré, Sjögren's syndromes and other inflammatory demyelinating polyneuropathies (Albers and Kelly, 1989; Pentland and Donald, 1994), in non-vasculitic steroid-responsive mononeuritis (Logigian et al., 1993), and in conditions that damage the nerve's blood vessels (i.e. epi- and endoneurial vasculitis) (Said, 1995). Acute neurotoxic insults like those produced by anti-neoplastic and anti-HIV chemotherapy also present with neuropathic pain. Neuropathic pain in cancer patients is often present when tumors invade, compress or stretch peripheral nerve (Diaz-Arrastia et al., 1992; Portenoy, 1992), and even in cases where there does not appear to be tumor-evoked nerve damage (i.e.

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paraneoplastic sensory neuropathy) (Smitt and Posner, 1995).

In the usual case, the neuropathy presents with both structural damage to primary afferent axons and their ensheathing glia, and an accompanying inflammatory process (a neuritis) that involves mobilization of the immune system. This obviously occurs when the nerve damage involves an infection (as in herpes zoster) or an autoimmune reaction (as in Guillain–Barré syndrome). However, it also occurs in the absence of infection when there is injury to any of the cell types that compose the peripheral nerve, because cellular debris is an immune stimulus. For example, inflammation occurs whenever there is axonal or myelin degeneration, as in acute nerve trauma (transection, crush, stretch, ischemic and radiation injuries) and acute neurotoxic insults.

The neuronal and glial degeneration, and microvascular damage, seen in diabetes may evoke a chronic, or chronically recurrent, immune response (Said et al., 1997). The nerve's axons are exposed to an inflammatory milieu even when the inflammation involves only the nerve's blood vessels, as in some vasculitic conditions, or when only the perineurium is inflamed (i.e. a perineuritis) (Asbury et al., 1972; Bourque et al., 1985). Moreover, sensory axons may be exposed as 'innocent bystanders' to inflammatory mediators released from malignant cells, or from immune cells attacking a malignancy (e.g. the endoneurial infiltration of cancer cells in neurolymphomatosis, or the exposure of a nerve root to the inflammatory milieu around a bony metastasis in a vertebral body).

The role of the immune response in the production of neuropathic pain is difficult to determine when it presents together with structural damage to primary afferent axons and their associated glia. We describe here an experimental neuritis of the rat's sciatic nerve that produces neuropathic pain sensations in the ipsilateral hind paw while producing no more than trivial structural damage to the nerve's axons and glia. A preliminary report has appeared (Eliav et al., 1996).

2. Methods

These experiments were performed according to a protocol that was approved by the NIDR Animal Care and Use Committee and in accordance with Federal law, the regulations of the National Institutes of Health and the guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

2.1. Subjects and surgery

Adult (250–350 g) male Sprague–Dawley rats (Harlan Sprague–Dawley Inc., Indianapolis, IN; Frederick, MD breeding colony) were used. Under sodium pentobarbital anesthesia (45 mg/kg, i.p., supplemented as necessary), the common sciatic nerves were exposed at the mid-thigh level by blunt dissection through the biceps femoris and gently separated from adjacent tissue. On one side, the nerve was wrapped in a band (approx. 3 mm wide and 25 mm long) of sterile hemostatic oxidized cellulose (Oxycel™; 'cotton' type; Parke-Davis & Co., Detroit, MI). The Oxycel was applied by passing curved forceps beneath the nerve (taking particular care to avoid stretching the nerve), grasping one end of the band and pulling it under the nerve. The end that was grasped was then gently folded over the nerve, the other end was folded over in the opposite direction and the excess was trimmed away. The Oxycel is intended to act as a sponge. It is wrapped loosely around the nerve and does not cause any nerve constriction. In one group of animals we saturated the Oxycel with 150 μl of undiluted modified Complete Freund's adjuvant (CFA) (0.1% heat-killed *Mycobacterium butyricum*, 15% mannide monooeate emulsifier and 85 % Drakeol 5 NF (a light mineral oil); Calbiochem, La Jolla, CA; catalog #344289), and in a second group with 150 μl lambda carrageenan (CARRA) (40 mg/ml in sterile saline; Sigma Chemical, St. Louis, MO). In both the CFA and CARRA groups the opposite nerve was exposed and wrapped with Oxycel saturated with 150 μl sterile saline.

In a third group, an experimental myositis was created by implanting Oxycel, saturated with 150 μl CFA, into a pocket made in the biceps femoris muscle at a position adjacent to where the nerve was treated. Oxycel, saturated with 150 μl sterile saline, was implanted in the opposite muscle. This group was a control for the effect of non-neuritis pain in the thigh and for the possible effects of a systemic immune response. Two other control groups were examined. In one, the sciatic nerve on one side was exposed and wrapped with Oxycel saturated with 150 μl sterile saline (the opposite side was not operated). The last control was a 'no surgical treatment' group whose rats were anesthetized but not operated on either side.

2.2. Behavioral assays

For all groups, the rat's mid-plantar hind paws (sciatic nerve territory) were tested for mechano- and cold-allodynia, and for heat- and mechano-hyperalgesia prior to surgery and daily thereafter for 8 days. Heat-hyperalgesia was assayed with the paw withdrawal test. As described elsewhere (Bennett and Xie, 1988; Hargreaves et al., 1988; Bennett and Hargreaves, 1990), the rats stood upon an elevated glass floor and a movable radiant heat source beneath the floor was aimed at the mid-plantar hind paw. Stimulus onset activated a timer, controlled by a photocell, the hind paw withdrawal reflex interrupted the photocell's light, automatically stopped the timer and terminated the stimulus. The temperature of the glass on which the rats stood was adjusted to approximately 25°C. The light intensity was adjusted at the beginning of an experiment in order to produce latencies of approximately 10 s; the light intensity was held constant thereafter. We express the data as difference scores, computed by subtracting the latency of

the control side (depending on the group, this was the side that was treated with saline, or the side that was not operated) from that of the treated side. In the 'no treatment' group, the left side was subtracted from the right. Negative difference scores thus indicate the presence of hyperalgesia; the normal difference score is zero (Bennett and Xie, 1988). Mechano-hyperalgesia was assayed with the pin-prick test. As described previously (Tal and Bennett, 1994), the rat was placed on an elevated, perforated floor and the tip of a safety pin was pushed slowly against the mid-plantar hind paw until the skin was dimpled, but not penetrated. The duration of the pin-prick evoked nocifensive withdrawal reflex was timed with a stopwatch. Normal responses are usually of very small amplitude and are too quick to time accurately by hand. We arbitrarily assigned normal responses a duration of 0.5 s The data are expressed as difference scores, computed by subtracting the withdrawal duration of the control side from that of the treated side. Positive difference scores thus indicate the presence of mechano-hyperalgesia (the normal difference score is zero). Mechano-allodynia was tested with von Frey hairs as described previously (Tal and Bennett, 1994). With the rat placed on the perforated floor, the hairs were tested in order of increasing stiffness, with each applied five times at intervals of 1–4 s to slightly different loci. The last hair to evoke at least one response was designated the threshold. We express the data by first ranking the von Frey hairs that were used from the standard Semmes-Weinstein series (Stoelting Inc., Wood Dale, IL) from smallest (rank #1 = 2.83 $1\log_{10}$ mg (0.07g) of force) to largest (rank #14 = 5.88 $\log_{10}$ mg (76 g) of force; with rank #15 = no response). Difference scores were computed by subtracting the rank of the threshold hair of the control side from that of the treated side. Negative difference scores thus indicate the presence of mechano-allodynia, the normal difference score being zero. Cold-allodynia was tested by an acetone spray test modified from that described by Choi et al. (Choi et al., 1994). With the rats standing upon the perforated floor, 250 μl of acetone was squirted onto the mid-plantar skin. The duration of the withdrawal evoked by the evaporative cooling was timed with a stopwatch. Rats that did not withdraw from the cold stimulus were assigned a score of 0 s. Difference scores were computed by subtracting the withdrawal duration of the control side from that of the treated side. Positive difference scores thus indicate the presence of cold-allodynia (the normal difference score is zero).

2.3. Drug testing

The effects of morphine and of the *N*-methyl-D-aspartate (NMDA) receptor antagonist, MK-801, were examined in CFA-treated rats using the heat-hyperalgesia, mechano-allodynia, and mechano-hyperalgesia assays (cold-allodynia was judged to be too variable and of insufficient severity for efficient testing). The experimenter was blind as to drug condition.

For MK-801, the rats were tested on post-operative day (POD) 3 to confirm the presence of the neuritis-evoked neuropathic pain and then injected intrathecally (i.t.) by the method of Mestre et al. (Mestre et al., 1994) with 10 μg MK-801 or saline (each 10 μl) and tested again 1 and 2 h later. The effect of MK-801 on heat-hyperalgesia was tested in one group of rats ($n = 8$) and a separate group ($n = 8$) was used to examine the effect on mechano-hyperalgesia and mechano-allodynia (the time required to complete all three behavioral assays is too long to determine the drug effect at a fixed post-injection time point). The MK-801 experiment was repeated once ($n = 8$/group), and a third experiment was done using 20 μg MK-801 ($n = 8$/group).

For morphine, rats were tested on POD 3 to confirm the presence of neuropathic pain, then injected with morphine (10 mg/kg, subcutaneous injection between the scapulae), tested 50 min later and finally injected with naloxone (1 mg/kg, i.p.) and tested 30 min later. The effect of morphine on heat-hyperalgesia was examined in one group of animals and the effects on mechano-hyperalgesia and mechano-allodynia were examined in a separate group ($n = 7$–8/group).

2.4. Data analysis

For each treatment group, the difference scores from the heat-hyperalgesia, mechano-hyperalgesia and cold-allodynia tests were analyzed with repeated measures ANOVA followed by pair-wise comparisons between baseline and post-operative scores using the Fisher PLSD test. For the mechano-allodynia data (rank difference scores), pair-wise comparisons to pre-treatment baseline values were made with non-parametric Wilcoxon signed-ranks matched-pairs tests.

For the drug studies, non-transformed data (response latencies or duration, and hair rank) were analyzed for the treated- and control-sides separately using paired *t*-tests for side-to-side comparisons and unpaired *t*-tests for comparisons between groups for the heat-hyperalgesia and mechano-hyperalgesia data, and Wilcoxon signed-ranks matched-pairs tests for the mechano-allodynia data.

2.5. Histology

For the light-microscopic analysis of plastic-embedded sections, three CFA-treated rats were sacrificed with an overdose of sodium pentobarbital on POD 3–4 (the approximate time of peak symptom severity) and perfused transcardially with ice-cold phosphate-buffered saline (PBS) to exsanguination, followed by 200 ml of fixative (3% paraformaldehyde, 3% glutaraldehyde, and 0.1% picric acid in 0.1 M cacodylate buffer (pH 7.4)). Pieces of the sciatic nerve from both sides were harvested from the treated regions, post-fixed overnight, osmicated and embedded in Epon. Sections (1 μm) were stained with toluidine blue.

For immunocytochemistry, three CFA-treated rats were sacrificed with an overdose of sodium pentobarbital on POD 3 and perfused transcardially with ice-cold saline followed

by 200 ml of 4% paraformaldehyde in PBS. Pieces of the sciatic nerve (approximately 1 cm long with the treated region at the center) were harvested from both sides and post-fixed overnight in 10% formalin. Following cryoprotection in 20% sucrose in PBS, 10 μm frozen sections were cut and thaw-mounted onto gelatinized slides and stained with hematoxylin and eosin (H&E). Alternate sections were used for the immunocytochemical demonstration of the CD4 and CD8 T-cell markers using a modification of a procedure described previously (Herzberg et al., 1995). Briefly, sections were incubated for 1 h at room temperature with 5% normal goat serum (Life Technologies, Gaithersburg, MD) and 10% Fc-receptor blocker (Accurate Chemical and Scientific, Westbury, NY) in PBS. All subsequent incubations included 5% normal goat serum and 0.1% Triton X-100 (Sigma). Sections were incubated overnight at 40°C in a closed, humidified chamber with a 1:500 solution of monoclonal mouse anti-rat-CD4 or anti-rat-CD8 antibody (Pharmingen, San Diego, CA) in PBS. Following three washes in PBS, sections were incubated for 2 h at room temperature with a 1:200 solution of goat anti-mouse-IgG antibody (Cappel, Aurora, OH) conjugated to fluoroscein (CD4 sections) or rhodamine (CD8 sections). Slides were then washed three times with PBS, dehydrated in alcohol and cover slipped using Cytoseal 60 (Electron Microscopy Sciences, Ft. Washington, PA). Control sections were treated only with the secondary antibody, or with primary antibody that had been pre-absorbed with the appropriate antigen (rat CD4 or CD8 peptides) (Pharmingen).

3. Results

As expected, the pre-treatment baseline difference scores (Fig. 1) were not significantly different from zero in any of the four tests. Moreover, there were no significant between-group differences for the baseline scores. For all groups combined (with left and right hind paws averaged together), the baseline mean (±SEM) of the heat-evoked withdrawal latencies was 9.7 ± 0.12 s. The baseline mean (±SEM) of the duration of the pin prick-evoked withdrawal was 0.5 ± 0.0 s. The baseline mean (±SEM) of the duration of the cold-evoked withdrawal was 0.5 ± 0.1 s. The baseline median von Frey hair threshold corresponded to hair rank #11 (5.07 $\log_{10}$ mg; 12 g force).

3.1. Effects of the different treatments

CFA treatment produced statistically significant heat-hyperalgesia, mechano-hyperalgesia, mechano-allodynia and cold-allodynia on the ipsilateral hind paw (Fig. 1). The abnormal pain responses began on POD 2–3, reached peak severity on POD 3–4, and were resolved by POD 5–6, with normal responsiveness thereafter. The hind paws on the contralateral saline-treated side showed little or no change in responsiveness. CARRA treatment also produced statistically significant heat-hyperalgesia, mechano-hyperalgesia, mechano-allodynia and cold-allodynia on the ipsilateral hind paw, and these abnormalities had a time course like that seen with CFA treatment (Fig. 1).

The neuritis-evoked behavioral abnormalities were readily apparent in a large majority of the rats in the heat-hyperalgesia, mechano-hyperalgesia and mechano-allodynia tests. As shown for CFA treatment in Fig. 2, in the heat-hyperalgesia test, 83% of the rats had difference scores that were more extreme than the normal mean difference score by 2 or more units of standard deviation. The same was true for 95% of the rats in the mechano-allodynia test and for 80% of the rats in the mechano-hyperalgesia test. However, cold-allodynia was typically not severe and was clearly present in only about 50% of the animals (Fig. 2).

Unlike the behavior seen in CCI rats (Bennett and Xie, 1988), neither CFA nor CARRA treatment caused foot-drop, hind paw eversion or ventroflexion of the toes, indicating that there was no significant damage to motor axons. Moreover, while CCI rats nearly always walk with a severe limp during the first week after nerve injury, CFA- and CARRA-treated rats either did not limp or limped infrequently.

The experimental myositis produced no significant change in any of the pain tests (Fig. 1). Neither the group with unilateral exposure to Oxycel saturated with saline (with no operation contralaterally) nor the 'no surgical treatment' group demonstrated any abnormal pain responses (Fig. 1), with the single exception of a statistically significant decrease in sensitivity to noxious heat on the first day following unilateral exposure to Oxycel/saline.

3.2. Histology

Gross examination of the three animals sacrificed 3–4 days after CFA treatment (the approximate time of peak symptom severity) revealed a nerve of normal diameter. The lymph nodes in the popliteal fossa were markedly enlarged on the side of treatment.

Light microscopic examination of toluidine blue-stained 1 μm sections taken through the treated region of nerve (Fig. 3) revealed no more than three to four degenerating profiles in two of the three cases. In the third case, there was a patch of about two dozen demyelinating axons just beneath the epineunum. The nerves from all animals exhibited greater than normal space between axons, suggesting a mild edematous reaction. Numerous immune cells were found in all nerves, including easily recognizable macrophages, lymphocytes and granulocytes. Examination of the 10 μm frozen sections stained with H&E revealed similar findings.

Immunocytochemical staining for T-cell lymphocytes bearing the CD4 and CD8 markers revealed identical staining patterns for both types of cells (Fig. 4). Very heavy staining was seen along the outside of the epineurial sheath, as might be expected because this was where the inflamma-

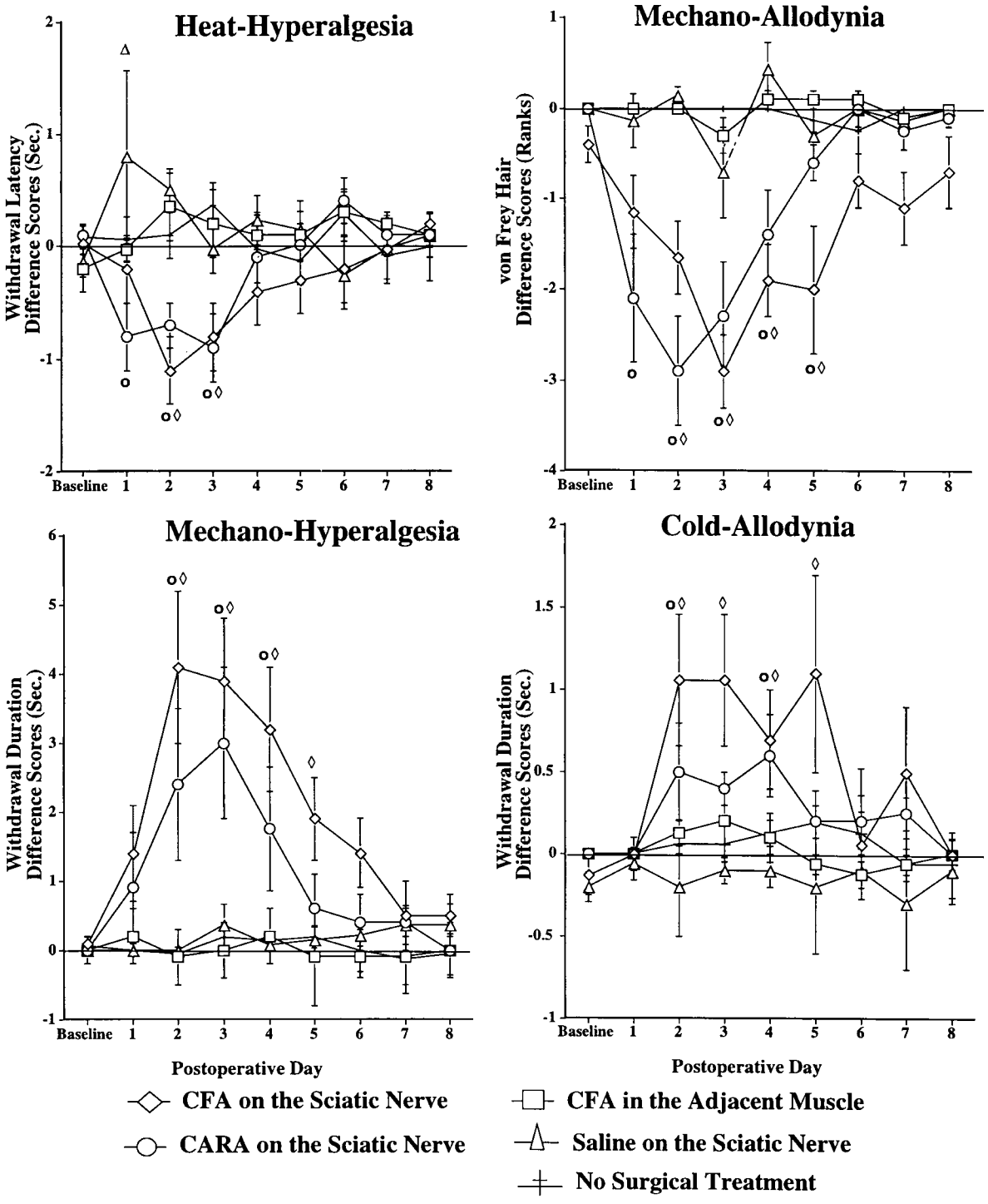

Fig. 1. The effects of CFA and CARRA treatment compared to three control conditions (CFA applied to muscle = myositis, saline applied to the nerve, and no treatment; n = 8/group) for heat-hyperalgesia, mechano-allodynia, mechano-hyperalgesia and cold-allodynia. For each test, the data are presented as mean ± SEM difference scores. Note that negative difference scores indicate the presence of heat-hyperalgesia and mechano-allodynia, whereas positive difference scores indicate mechano-hyperalgesia and cold-allodynia. As expected, for all tests difference scores in the pre-treatment baseline examinations were not significantly different from the normal difference score of zero (horizontal line). Symbols above the error bars (open diamonds: CFA; open circles: CARRA; open triangle: saline treatment) denote difference scores that are significantly different from normal ($P < 0.5$). Note that only CFA and CARRA treatment evoke hypersensitive pain responses. There was a single statistically significant time point of hypoalgesia on POD 1 following saline treatment in the heat-hyperalgesia test.

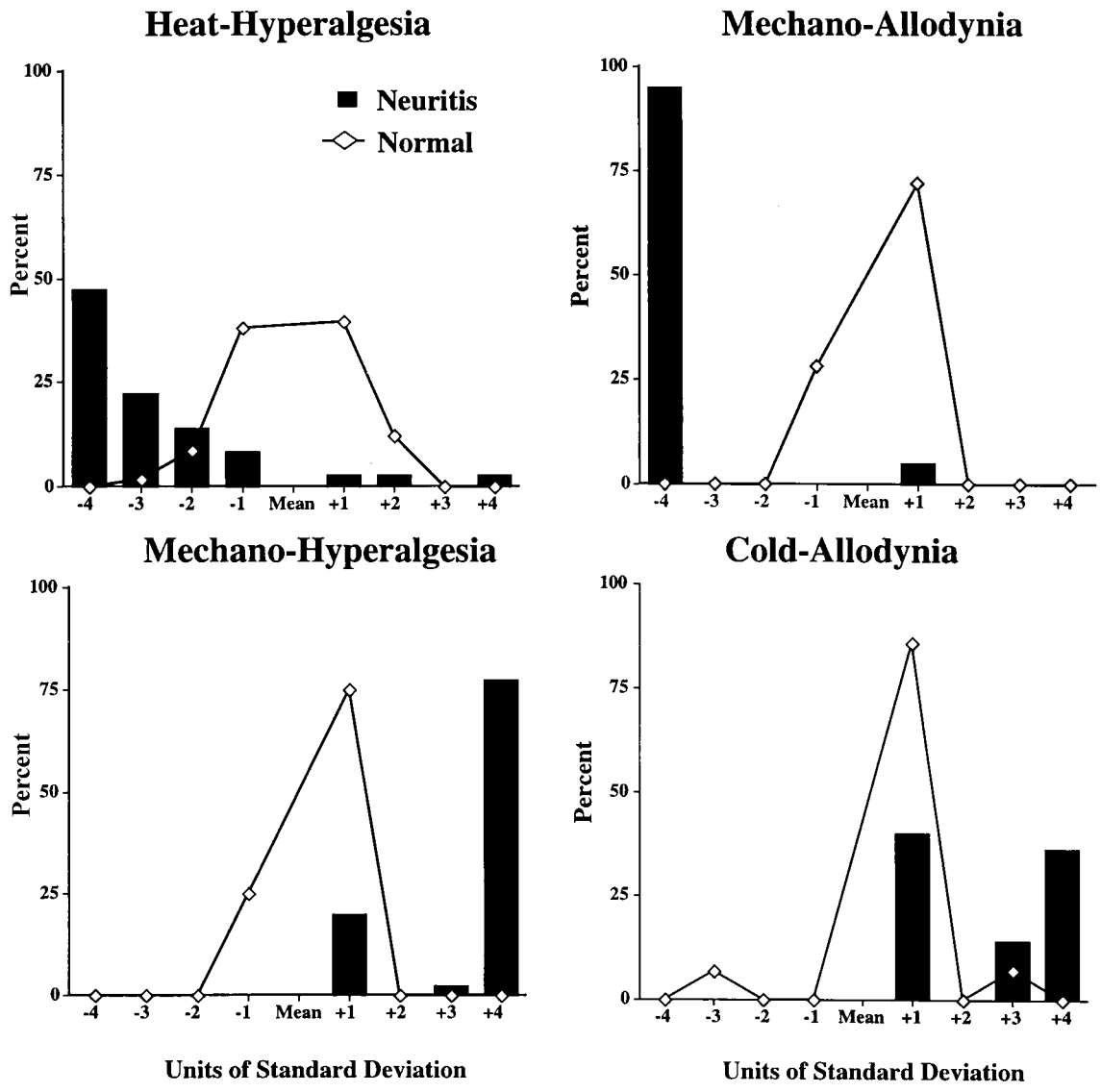

Fig. 2. Distributions of difference scores, categorized in units of standard deviation from a sample of normal animals. Normal control values from pre-treatment baseline measures are shown by the solid lines with open diamonds, the abscissa shows the mean and standard deviations of the normal distributions, the ordinate shows the percentage of scores falling in each standard deviation category. Data from animals with the experimental neuritis were obtained on POD 3 following CFA treatment (filled bars) and are plotted relative to the normal distribution. A large majority of the animals with the neuritis have clearly abnormal scores that are equal to or greater than 2 SD from the control mean in the heat-hyperalgesia, mechano-allodynia and mechano-hyperalgesia tests, but only about one-half the cases have clear-cut cold allodynia. Note that negative difference scores indicate the presence of heat-hyperalgesia and mechano-allodynia, whereas positive difference scores indicate mechano-hyperalgesia and cold-allodynia. Data from all experiments completed to date. $n = 58$ for all control distributions; $n = 40$ for all post-treatment distributions, except for heat-hyperalgesia where $n = 38$.

tory stimulus was applied. However, there was also a very obvious population of immunoreactive T-cells in the endoneurial compartment (Fig. 4), and these had the size and simple, oval shape that is characteristic of lymphocytes. If the endoneurial lymphocytes had migrated across the epineurium, one might expect to find a gradient of cell density, with the highest concentration of cells beneath the epineurium. This was definitely not the case, and it thus appears very likely that the infiltrating cells arrive via the endoneurial vasculature. Nerves contralateral to the CFA-treated side, nerves treated with saline and nerves taken from the animals with the myositis did not show staining for CD4 or CD8 T-cells. Omission of the primary antisera incubation and pre-incubation of the working dilution of the primary antisera with CD4 or CD8 peptide eliminated all staining in CFA-treated nerves.

3.3. Effects of morphine

Statistically significant mechano-allodynia, mechano-hyperalgesia and heat-hyperalgesia were present in all groups prior to morphine injection.

For mechano-allodynia, morphine had a significant analgesic effect on both the neuritis and sham-treated

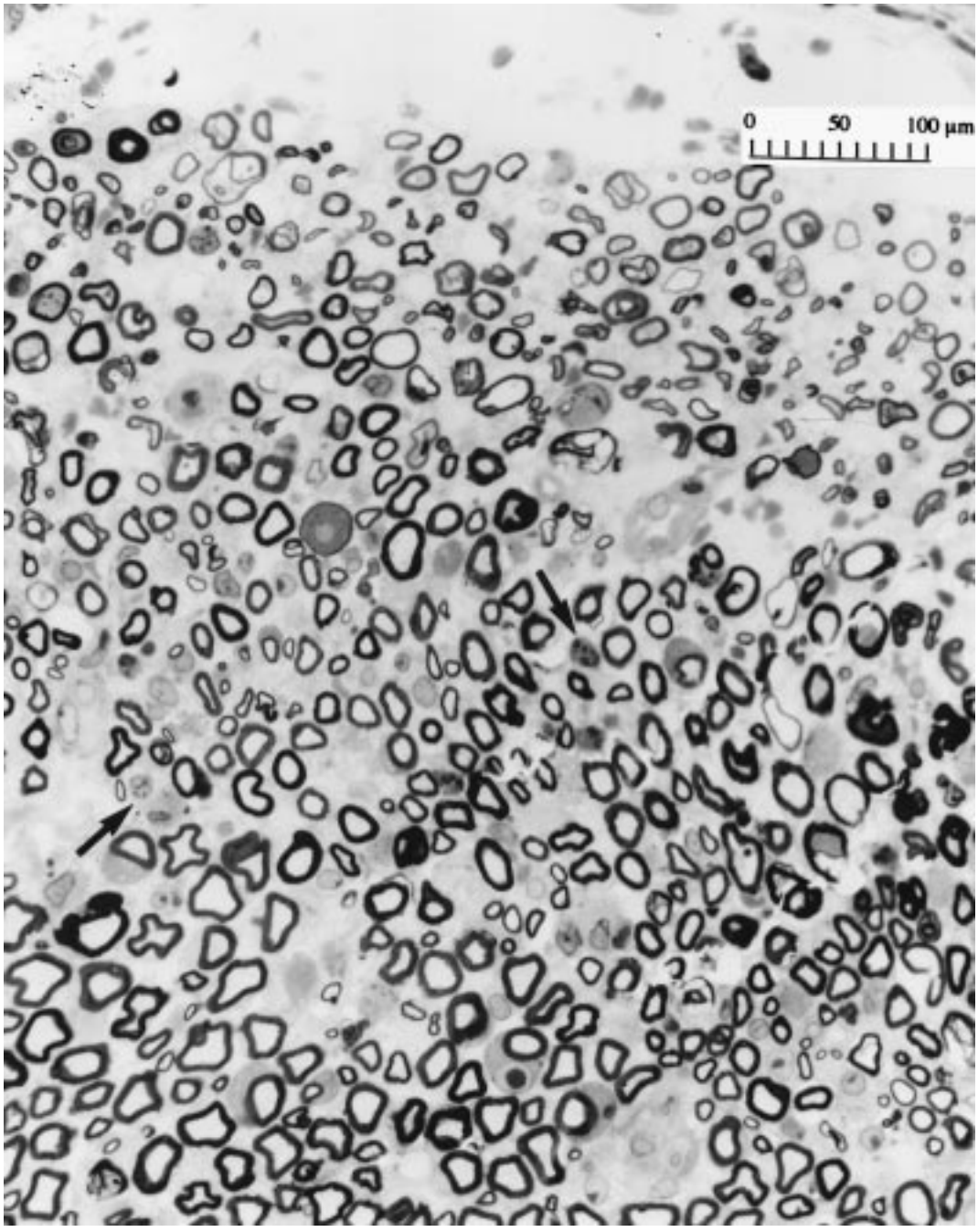

Fig. 3. Light photomicrograph of a 1 µm plastic-embedded section of sciatic nerve stained with toluidine blue. The section is from the center of the treated region, 3 days postoperative. There are no clear examples of degenerating axonal profiles. Note the greater than normal inter-axonal spacing (presumably due to endoneurial edema) and the many immune cells (two neutrophils are marked by arrows).

sides (Fig. 5). However, the difference scores were relatively unaffected, i.e. the CFA-treated side was still significantly hyper-responsive relative to the sham-treated side. The morphine effect was completely reversed by naloxone, without any significant change in the difference scores. Saline injection had no effect on mechano-allodynia, nor did the injection of naloxone following saline treatment.

For mechano-hyperalgesia, morphine had a statistically significant effect on response duration on the side of the neuritis; and this was completely reversed by naloxone. There was no significant depression of the response duration on the sham-treated side. This apparent lack of effect may be due to a 'floor effect', i.e. the normal response to pin-prick is of such small magnitude and brief duration prior to morphine that a decrease can not be demonstrated. However, what one might expect from morphine in the

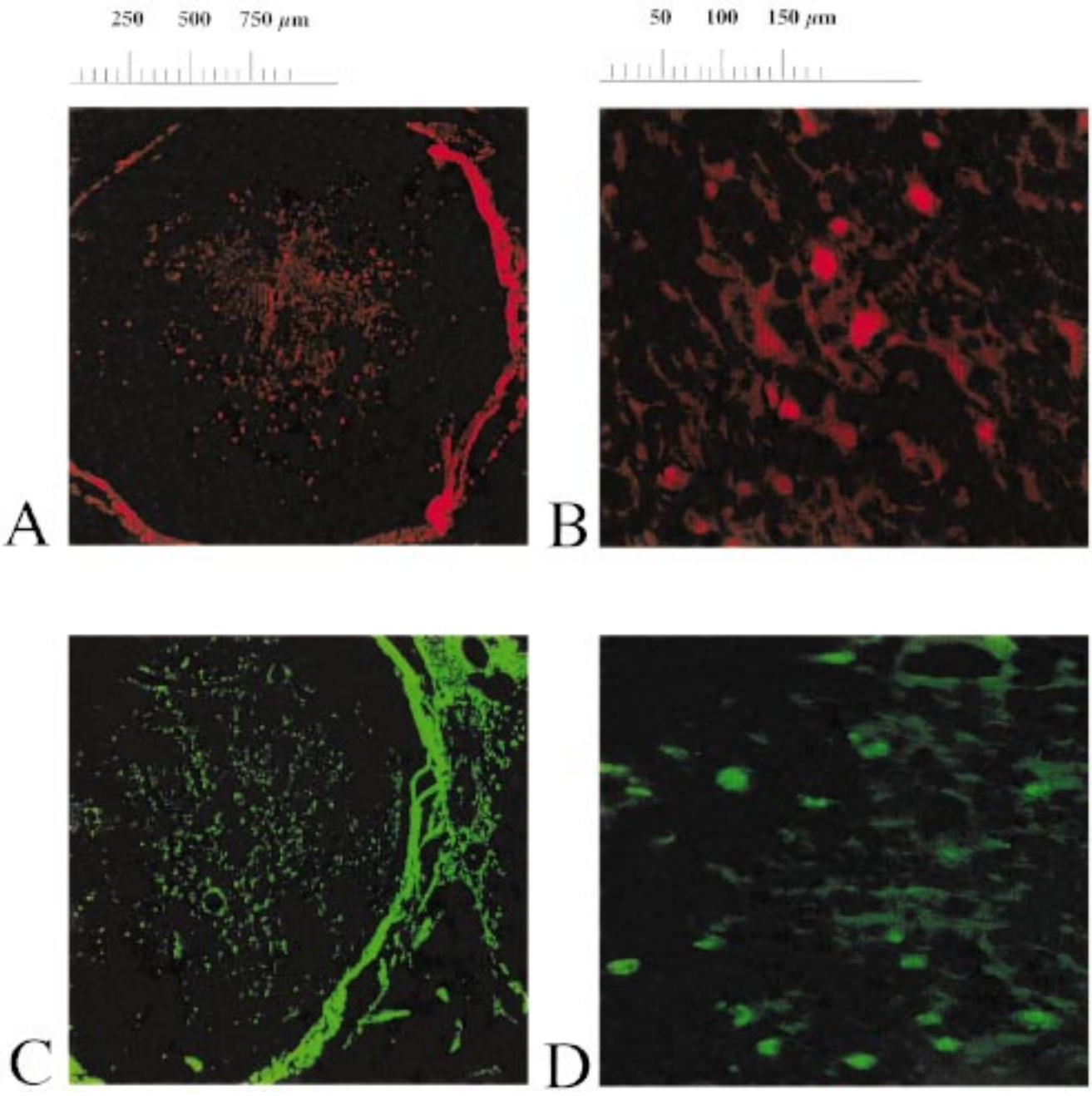

Fig. 4. Cross sections of CFA-treated sciatic nerves stained immunohistochemically for T-cells bearing the CD4 (green) and CD8 (red) markers. In the low magnification views (A and C), note the intense staining over the epineurium and the stained cells scattered throughout the endoneurial compartment. Note also the absence of any outside-to-inside gradient for the density of the stained cells. The stained cells (B and D) had the size and simple, oval shape characteristic of lymphocytes.

normal case is that the animal would have no response at all to pin-prick. In three of seven cases following morphine, pin-prick failed to evoke any response on the side contralateral to the neuritis (such non-responses were never seen on either side before morphine or after naloxone in either the morphine- or saline-treated groups). Saline injection had no effect on mechano-hyperalgesia, nor did the injection of naloxone following saline treatment.

For heat-hyperalgesia, morphine had a significant analgesic effect on both the neuritis- and sham-treated sides. The mean difference score appeared to be relatively unaffected by morphine, but variability increased, and the post-morphine mean difference score was no longer significantly different from zero. Following naloxone, the mean difference score was again significantly different from zero. Post-naloxone, both sides appeared to be slightly hypersensitive relative to their pre-injection baseline levels of heat-hyperalgesia, but this was statistically significant only for the

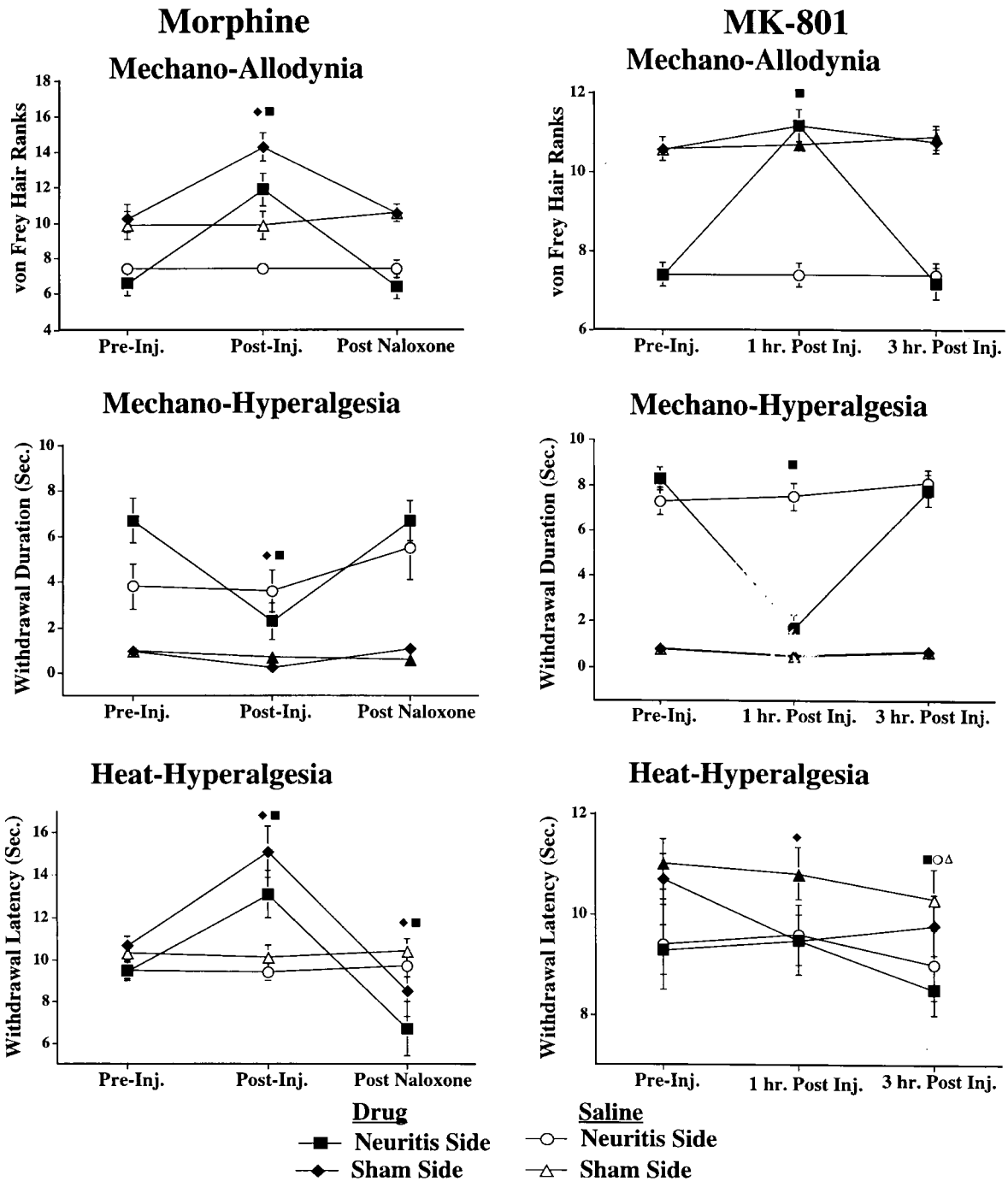

Fig. 5. Effects of morphine (left-hand column) and MK-801 (right-hand column) on neuritis-evoked mechano-allodynia (mean ± SEM hair ranks), mechano-hyperalgesia (mean ± SEM withdrawal duration), and heat-hyperalgesia (mean ± SEM withdrawal latency), determined three days after CFA treatment. Small symbols denote significant differences relative to pre-injection values. Left: One group of CFA-treated rats (filled symbols) received a subcutaneous injection of morphine (10 mg/kg) immediately after pre-injection baseline testing, the other group (open symbols) was treated identically except that the injection was saline. $n = 8$/group. Both groups were tested 50min later and again 30 min after an i.p. injection of naloxone (1 mg/kg). Pre-injection baseline tests showed significant ($P < 0.5$) mechano-allodynia, mechano-hyperalgesia, and heat-hyperalgesia in both groups. Saline injection had no significant change in any of the tests. Note that in the graph for mechano-hyperalgesia the curves showing the sham-side responses for the morphine- and saline-treated groups are nearly superimposed. Right: One group of CFA-treated rats (filled symbols) received an intrathecal injection ofMK801(10 μg/10 μl) immediately after pre-injection baseline testing, the other group (open symbols) was treated identically except that the injection was saline. $n = 16$/group (replicate experiments combined). Both groups were tested 1 and 2 h post-injection. Pre-injection baseline tests showed significant ($P < 0.5$) mechano-allodynia, mechano-hyperalgesia, and heat-hyperalgesia in both groups. Note that in the graph for mechano-hyperalgesia the curves showing the sham-side responses for the morphine- and saline-treated groups are superimposed. Note also that for heat-hyperalgesia the decrease seen at 1 h post-injection is for the sham-treated side (no change in the neuritis side) and that withdrawal latencies are significantly shorter at the 2 h post-injection test for both groups (see Discussion).

sham-treated side. Saline injection had no effect on heat-hyperalgesia, nor did the injection of naloxone following saline treatment.

3.4. Effects of MK-801

Statistically significant mechano-allodynia, mechano-hyperalgesia and heat-hyperalgesia were present in all groups prior to MK-801 injection (Fig. 5). Tests using 10 μg i.t. MK-801 were run in duplicate and the results were statistically indistinguishable for the mechano-allodynia, mechano-hyperalgesia and heat-hyperalgesia tests. The results were therefore pooled and the data presented are based on $n = 16$/group. The results from the replication using 20 μg i.t. MK-801 were nearly identical to those obtained with 10 μg i.t. MK801 (data are not shown).

For mechano-allodynia, MK-801 completely reversed the hypersensitivity on the neuritis side, but had no effect on the sham-treated side, i.e. there was a specifically anti-allodynic, rather than an analgesic effect. MK-801's effect was completely dissipated 3 h after injection. Intrathecal injection of saline had no effect on mechano-allodynia.

For mechano-hyperalgesia, MK-801 completely reversed the hypersensitivity on the neuritis side (the difference score was not significantly different from the normal difference score of zero). MK-801 had no effect on the average response duration obtained on the sham-treated side, and it did not cause any animals to become 'non-responders' on either side. The drug's effect on the neuritis side was completely dissipated 3 h after injection. Saline injection had no effect on mechano-hyperalgesia.

For heat-hyperalgesia, MK-801 produced a statistically significant reduction of latencies on the sham-treated side, but had no effect on the side of the neuritis. This unexpected result was seen in both replicates using 10 μg and in the experiment (data not shown) using 20 μg. At the 2 h post-injection test in the MK-801-treated groups, the difference score was again statistically significant, but there was a significant decrease (relative to pre-injection baseline) in the latencies obtained from both sides. We have no explanation for these findings. The interpretation is complicated by changes seen in the saline-treated group, where there was also a significant decrease (relative to pre-injection baseline) in the latencies obtained from both sides at the 2 h post-injection test.

4. Discussion

We have shown that an inflammation of the sciatic nerve at the level of the mid-thigh produces neuropathic pain sensations in a distant region (the ipsilateral hind paw). We believe that 'neuropathic' is the most useful term for the abnormal pains that can be evoked from the hind paw. We favor the term because of its general sense of 'pain due to dysfunction of the nervous system', but we note that it is used here in a new context. We have considered the alternative terms 'neuritic' and 'neuralgic' pain, but have rejected both because they do not differentiate pain at the site of inflammation (a sore nerve) from the hyperalgesia and allodynia seen in the hind paw. We think that it is highly probable that an inflamed nerve is sore locally (nociceptive pain because the nociceptors of the nervi nervorum are activated), but that the pains evoked from the hind paw are produced by a different mechanism.

The neuropathic pain produced by the neuritis is accompanied by no more than minor structural damage to axons or glia. It is specific to a neuritis, rather than a generalized effect due to pain in the upper leg or to systemic exposure to an immune stimulus (Watkins et al., 1995), because the same amount of CFA placed in muscle produced no change in pain responsiveness. The effect is not due to simple nerve irritation or to post-surgical pain emanating from the incision, because unilateral Oxycel/saline treatment had no effect. Application of CFA to the surface of the nerve evoked an endoneurial inflammation characterized by evidence for plasma extravasation and the infiltration of immune cells.

The neuritis-evoked mechano-allodynia responded in 'neuropathic' fashion to pharmacological challenge. It was specifically inhibited by NMDA receptor blockade and relativelv resistant to the dose of morphine tested (10 mg/kg). However, the relative ineffectiveness of morphine will need to be confirmed with an examination of its entire analgesic dose range.

Both morphine and MK-801 blocked the mechano-hyperalgesia, and it seems probable that both drugs had relatively specific anti-hyperalgesic, rather than analgesic, effects. However, we noted that on the side contralateral to the neuritis, morphine rendered three of seven animals insensate to pin prick. Such insensitivity was never seen on the neuritis side following morphine injection (nor on either side before morphine or after naloxone, nor on either side following MK-801 injection). We interpret this to mean that even the high dose of morphine (10 mg/kg, s.c.) used here has relatively little effect on the normal response to pin-prick (on the side contralateral to the neuritis, four of seven cases did respond). This is in contrast to morphine's very strong effect on the neuritis side where the hyperalgesic responses were almost normalized. It seems likely that the normal response to pin-prick is due to input from Aδ-nociceptors, and it is known that the pain evoked by Aδ input is relatively resistant to morphine. It is possible that the hyperalgesic response to pin-prick on the neuritis side is to due input from C-nociceptors; pain arising from C-fiber input is known to be relatively susceptible to morphine (Dickenson and Sullivan, 1986; Yeomans et al., 1996).

Heat-evoked responses were blunted on both sides by morphine, the results suggesting that this analgesic effect did not eliminate the relative hypersensitivity on the neuritis side. MK801's effects on heat-hyperalgesia were complex and puzzling. The drug's effect may have been obscured by increased sensitivity due to repeated testing. However, we

think this is an unlikely explanation because in our hands very similar experiments involving repeated testing in CCI have always yielded clear-cut results (Xiao and Bennett, 1994, 1995, 1996; Imamura and Bennett 1995;). Additional experiments will be needed before MK-801's effects on neuritis-evoked heat-hyperalgesia can be understood.

Minor structural damage to axons and glia was present in at least some cases. We think it is very unlikely that this degree of structural damage can account for the neuropathic pain. First, the pain lasted for only a few days. Second, the animals recovered to a state of normal responsiveness with no sign of a persistent sensory deficit and at no time was there any evidence of muscle weakness (no foot-drop, ventroflexed toes, or hind paw eversion). Third, the brief duration of the abnormal pain state produced by the neuritis is in marked contrast to the long-lasting pain states seen in models that produce substantial structural damage (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992; DeLeo et al., 1994; Na et al., 1994). Fourth, we have seen that briefly painting the nerve sheath with alcohol in order to destroy the nervi nervorum (see below) produces a very marked thin ring of subepineurial degeneration that involves a far greater number of axons than what was seen with the neuritis. But animals with this subepineurial degeneration did not develop abnormal pain responses. We conclude that the abnormal pain responses of the neuritis animals were not due to structural damage, but rather to a neuroimmune effect (as discussed below). We note, however, that the hypothesized neuroimmune effect is likely to be accompanied by subtle structural alterations; for example, the vacuolation of the outermost lamellae of the myelin sheath that is seen after tumor necrosis factor-alpha (TNFα) treatment (Wagner and Myers, 1996a).

We have also considered the possibility that our results are due to only the epineurial component of the inflammatory response, i.e. to the activation of the nociceptive innervation of the nerve sheath, the nervi nervorum (Bove and Light, 1997). The amount of nociceptor activity generated from this source is probably quite small because the sheath's innervation is relatively sparse. Although we did not test for pain in the upper leg in the myositis animals, it seems certain that this region was sore (Kehl et al., 1996). The amount of C-nociceptor activity generated by the experimental myositis was probably much greater than that emanating from the nervi nervorum, but the myositis did not generate abnormal pain in the hind paw. However, arguments based on the amount of nociceptor activity must be balanced against the evidence that shows that nociceptors from different tissues have considerably different potencies in evoking the NMDA receptor-mediated central hypersensitivity state (Woolf and Wall, 1986). We have performed preliminary experiments (unpublished results) in which the nervi nervorum were destroyed by a brief topical application of alcohol (this also produced damage to axons in a thin ring just beneath the epineurium). Denervation of the nervi nervorum by itself did not produce any alteration of pain responsiveness on the ipsilateral hind paw. Rats with a denervated nervi nervorum that were subsequently treated with CFA developed an unaltered neuritis-evoked neuropathic pain syndrome. While these results need to be confirmed, it seems likely that activation of the nervi nervorum is not essential for the neuropathic pain syndrome described here.

Our results indicate that a focal, purely inflammatory reaction in or around a nerve (a neuritis) can give rise to neuropathic pain sensations in a distant region. This idea has several clinical implications. (1) An acute episode of neuritis-evoked neuropathic pain may contribute to, or be an essential prerequisite for, the genesis of chronically painful peripheral neuropathies that arise when there is also structural damage to the nerve. The severity of the initial inflammatory response may vary greatly from person-to-person, or from time-to-time, and this may explain why only a minority of cases of nerve damage develop a chronically painful neuropathy. The acute episode of neuritis-evoked pain may prime the system for more slowly developing pathogenic mechanisms; for example, the onset of ectopic spontaneous discharge in axotomized nociceptors. (2) A chronic, or chronically recurrent, neuritis due to infection, metabolic or vascular dysfunction, or even simple mechanical irritation of the nerve, may produce chronic neuropathic pain in the absence of significant (and clinically detectable) neural degeneration. This possibility may be of particular importance for Complex Regional Pain Syndrome Type I (reflex sympathetic dystrophy) where, by definition, there is pain without evidence of structural nerve damage. (3) A disease process near a nerve (or root) may secondarily involve it in an inflammatory milieu ('innocent bystander' effect) and thereby give rise to neuritis-evoked neuropathic pain. This hypothesis may be of particular importance for the enigmatic pain of paraneoplastic sensory neuropathy. An innocent bystander effect may also be of importance in cases of radicular pain where there is leakage of the nucleus pulposus, which is a very potent inflammatory stimulus (Olmarker et al., 1995).

We think it is most likely that a neuroimmune interaction in the endoneurial compartment plays a key role in producing the neuritis-evoked neuropathic pain. The first evidence that this might be true came from the experiments of Maves et al. (Maves et al., 1993, 1994) where chromic gut ligatures were placed very loosely around, or simply adjacent to, the sciatic nerve and the rats subsequently developed a short-lived (about 5–10 days) heat-hyperalgesia (but no mechano-allodynia or mechano-hyperalgesia) in the ipsilateral hind paw. The ligatures that they tied did not produce the nerve constriction seen with the Bennett and Xie (Bennett and Xie, 1988) procedure, and their photomicrographs showed that at least some of their cases had little or no structural damage in the endoneurial compartment. Chromic gut sutures evoke an inflammatory response (although this is probably less severe than that evoked by CFA or CARRA). In subsequent work it was shown (Maves

et al., 1995) that continuous perfusion of acidified saline onto the surface of the sciatic nerve also produced heat-hyperalgesia (the only behavior tested). Although the appearance of the nerves treated in this way was not reported, it is highly probable that the treatment evoked an inflammatory response.

A rapidly accumulating body of evidence indicates that neuroimmune phenomena are involved in the production of neuropathic pain. A conspicuous inflammatory reaction, an immune cell infiltration and increased endoneurial levels of pro-inflammatory cytokines (including TNFα, interleukin-1β and interleukin-6) have been detected at the site of nerve injury in animal models of painful peripheral neuropathy (Sommer et al., 1993; Clatworthy et al., 1995; Daemen et al., 1996; DeLeo et al., 1996; 1997; Kruger et al., 1996; Sommer and Myers, 1996). It has been shown (Sorkin et al., 1997) that the application of TNFα onto the mid-thigh sciatic nerve of normal rats produces ectopic discharge in Aδ- and C-fiber primary afferents, including identified nociceptors. The mechanism of this effect is not known, but it is highly suggestive that TNFα forms ion-permeable channels when it is incorporated into membranes (Kagan et al., 1992). It was shown (Wagner and Myers, 1996a) that injecting TNFα or its second messenger, ceramide, into the sciatic nerve produces a short-lived (approx. 3 days) mechano-allodynia and heat-hyperalgesia. The 55-kDa receptor for TNFα is up-regulated at the site of injury in CCI rats (Wagner et al. 1996). In peripheral tissues, at the level of the afferent receptor terminals, TNFα produces mechano-hyperalgesia when injected subcutaneously and it excites nociceptors when injected into their receptive fields. Cutaneous inflammation is accompanied by increased levels of TNFα, and neutralizing anti-TNFα antibodies reduce inflamatory hyperalgesia (Cunha et al., 1992; Sorkin et al., 1997; Woolf et al., 1997). Sensory axons will be exposed to TNFα and other pro-inflammatory cytokines wherever there is nerve infection or injury. The sources of TNFα and other cytokines will include infiltrating immune cells, reactive Schwann cells and fibroblasts (Wagner and Myers, 1996b). Additionally, it has been shown that the systemic administration of antisera directed against GD2 ganglioside evokes mechano-allodynia and the appearance of ectopic 'spontaneous' discharge in nociceptive Aδ- and C-fiber primary afferent axons (Xiao et al., 1997). These effects are almost certainly due to an immune response that follows antibody–epitope binding. Finally, the immune response evoked by epineurial application of gp120, an HIV envelope protein, to the rat sciatic nerve evokes ipsilateral hyperalgesia and allodynia in the absence of significant axonal degeneration (Herzberg et al., 1998).

We present the following hypothesis to account for the neuritis-evoked neuropathic pain behaviors described here. The endoneurial infiltration of immune cells and the reactive responses of Schwann cells and fibroblasts expose Aδ- and C-nociceptor axons to TNFα, other pro-inflammatory cytokines and, perhaps, nerve growth factor. These substances (singly, or in combination) will produce ectopic discharge in unmyelinated nociceptor axons (and perhaps other fibers). Importantly, ectopic discharge originating within the nerve at mid-axon level will travel in both directions — towards the spinal cord (orthodromic) and towards the periphery (antidromic).

The orthodromic discharge in nociceptors will evoke ongoing ('spontaneous') pain. The orthodromic discharge in C-nociceptors will also evoke and dynamically modulate the central hypersensitivity state that is mediated (at least in part) by activation of NMDA receptors (Woolf and Thompson, 1991; Gracely et al., 1992). It is important to note here that if maintained for a long period of time, even very low levels of C-nociceptor input are able to evoke and maintain the central hypersensitivity state (Cervero et al., 1993). The central hypersensitivity state would be expected to contribute to all the abnormal stimulus-evoked pains, and might be the most important mechanism for mechano-allodynia.

The antidromic discharge in C-nociceptors may release neuropeptides like substance P and calcitonin gene-related peptide into the peripheral tissues. This may lead to a neurogenic inflammatory response that will include nociceptor terminal sensitization. Nociceptor sensitization might contribute importantly to heat- and mechano-hyperalgesia but it is not clear whether it would contribute to cold- and mechano-allodynia.

We note that the duration of symptoms seen in this model is considerably shorter than the expected duration of a CFA-evoked inflammatory response. We suspect that anti-hyperalgesic cytokines such as interleukin-l0 and leukemia-inhibitory factor (Banner et al., 1998) may contribute to the resolution of symptoms despite an ongoing inflammation.

Our hypothesis suggests novel therapy — immunosuppressive treatments ought to have efficacy against at least some kinds of neuropathic pain. There is evidence that this is true. The immunosuppression achieved with perineural corticosteriod injection relieves hyperalgesia and allodynia in CCI rats (Johansson and Bennett, 1997) and systemic injections of corticosteriods relieves the neuropathic pain of animals with the experimental neuritis (Baños and Bennett, in preparation). Thalidomide, which inhibits the release of TNFα (and perhaps other cytokines), blocks neuropathic pain in CCI rats (Sommer et al., 1998; Shiiba, Baños and Bennett, in preparation). Moreover, the anti-inflammatory cytokine, interleukin-10, decreases neuropathic hyperalgesia and the nerve injury-evoked increase in endoneurial levels of TNFα (Wagner et al., 1998).

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